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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Significant controversies exist on which therapy constitutes the optimal treatment for *prostate cancer*. A better understanding of the biologic mechanisms responsible for the uncontrolled growth and motility of *prostate cancer* cells is critical to devise novel therapeutic approaches. Considerable interest has recently focused on the role of integrins as signal transducing molecules that ultimately control cell invasion, metastasis, and proliferation. The area of research that focuses on the role of integrins in the modulation of prostate cell metastatic establishment and growth is "under-investigated", whereas a significant progress has been made on breast cancer in this field. The studies, nonetheless, are important because integrins, specifically the β 3 integrin, play a role in cancer cell proliferation, tumor growth, invasion, and metastatic spread. In *prostate cancer* epithelial cells β_3 is upregulated, suggesting that, similar to its activities described in other cancer cells, it may act as a "motility and growth modulator" in prostate cells. The objective of these studies is to demonstrate *in vivo* a direct role for β_3 integrin in *prostate cancer* pathogenesis. The findings that will originate from the proposed study will help identify novel target molecules for the development of rational therapeutic approaches to limit or prevent *prostate cancer*.

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integrins, cell adhesion, extraellular matrix, prostate cancer cell migration, cell signaling pathways

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Final Scientific Report

Role of $\alpha_v\beta_3$ Integrin in the Establishment and Growth of Metastatic Lesions in Prostate Cancer

The findings described in the Background section of the original proposal showed that: a) the β_3-integrin is expressed in primary prostatic adenocarcinoma and in its metastatic lesions to the lymph nodes and bone; b) expression of $\alpha_v\beta_3$ by prostate cancer cells increases migration in *vitro* by upregulating cdc2 levels as well as by activating FAK. We hypothesized that $\alpha_v\beta_3$ contributes to the establishment and growth of metastatic lesions *in vivo* through at least one of these mechanisms.

The primary objective of the proposal was to assess the contribution of $\alpha_v\beta_3$ to metastatic establishment and growth of prostate cancer cells. It was planned to: test the well-characterized LNCaP cell transfectants (β_3-LNCaP, β_6-LNCaP, and mock-LNCaP) directly in an *in vivo* model of bone metastases; investigate the effects of suppressing the endogenous expression of $\alpha_v\beta_3$ by antisense methods in PC-3 prostate cancer cells; examine the effects of ectopically expressing β_3 integrin in a third prostate cancer cell line (CWR22Rv1) that is known to form micrometastases *in vivo*. In addition, since the activation status of $\alpha_v\beta_3$ is important in metastatic establishment, we planned to examine the effects of ectopically expressing an activated form of β_3 in LNCaP and CWR22Rv1 cells.

The second aim of this proposal was to investigate whether inhibition of two identified pathways mediating migration in prostate cancer cells in response to $\alpha_v\beta_3$ expression (i.e., cdc2 and FAK) blocks metastatic establishment and growth *in vivo*. Furthermore, since the role of cdc2 kinase in cell migration is a novel discovery, we aim to identify the cyclin(s) associated with cdc2 that may contribute to cell migration

However, during this training period, much work was done in response to reviewers' comments of a manuscript we submitted for publication. Therefore, the chronological order of the tasks listed in the Statement of Work (SOW) were altered. Also, due to the departure of Dr. Languino to theUniversity of Massachusetts in August, 2002, the period of this grant was curtailed. Therefore, several of the tasks were not completed.

The key training accomplishments have been achieved through performing experiments and reading the literature which ultimately resulted in the attached paper, submitted to the Journal of Cell Biology. These include performing migration experiments, cell cycle analysis, and preparing cells for immunofluorescence.

The key research accomplishments have been:
1. The determination that cdc2 regulates migration in prostate cancer cells as well as other cell types;

2. The identification of cyclin B2 as the cdc2 co-factor in cell migration;
3. LNCaP cells expressing αvβ3 form larger tumors in the SCID-human bone model than LNCaP cells expressing αvβ6.

Below is a point by point summary of the training and research accomplishments done for each task in the SOW.

SOW Task 1. Investigate a role for αvβ3 in prostate cancer cell metastatic establishment and growth *in vivo* using SCID-human bone model (months 1-6):
1. Implant human bone fragments, wait 4 weeks
2. Inject cells, wait 6 weeks
3. Harvest implants, measure growth of tumors, perform statistical analysis.

The PI sent β3 –LNCaP, β6-LNCaP, and mock-LNCaP cells to our collaborator in this task, Dr. Cher, and they were tested in the SCID-human bone model. According to their analysis, no difference was seen between the β3 –LNCaP and mock-LNCaP, but β3 –LNCaP grew larger tumors than β6 –LNCaP.

SOW Task 2a. Establish stable CWR22Rv1 cell transfectants expressing β3 integrin, expressing β6 integrin, or vector only (months 1-3):
1. Transfect CWR22Rv1 cells
2. Select for stably-transfected cells
3. Expand clones
4. Screen clones by FACS analysis

Not done.

SOW Task 2b. Establish stable LNCaP and CWR22Rv1 cell transfectants expressing D723R β3 integrin (months 4-6):
1. Obtain or generate D723R β3 integrin in expression vector
2. Transfect CWR22Rv1 and LNCaP cells
3. Select for stably-transfected cells
4. Expand clones
5. Screen clones by FACS analysis

Not done.

SOW Task 3. Infection of various prostate cancer cells with adenoviral constructs (months 7-9):
1. Subclone cdc2dn and FAKdn cDNAs into adenovirus vector
2. Expand viruses
3. Infect cells with various titers
4. Prepare extracts after various time points
5. Establish expression of ectopic cdc2dnHA and FAKdn by immunoblotting
6. Infect cells with optimum titer
7. Perform migration assays with transduced cells at optimum time point

Not done.

SOW Task 4. Determine effect of cdc2dn on PC-3 migration (months 10-12):
1. Cotransfect PC-3 with pCMVbetagal and pCMVcdc2dn, pCMVcdc2wt, or vector only
2. Perform migration assays 4-24 hours; fix some cells for cell cycle analysis
3. Fix and stain for betagalactosidase activity
4. Count transfected cells on top and bottom of filter
5. Perform cell cycle analysis on cells used for migration

PC3 cells responded in a similar manner as LNCaP to ectopic expression of cdc2dn (reduced migration at 48 and 72 hours after transfection), but did not increase migration in response to ectopic expression of cdc2wt. However, increased migration in response to ectopic expression of cdc2wt was seen in HeLa cells 24 hours after transfection. Please see attached figure 5 (note: not in the attached paper).

SOW Task 5. Determine effect of pharmacological inhibitors on PC-3 migration (months 10-12):
1. Treat PC-3 cells with olomoucine and alsterpaullone for 24, 48, and 72 hours
2. Perform migration assays on treated and non-treated cells 4-24 hours
3. Fix, stain with crystal violet overnight
4. Count migrated cells

A more potent and specific inhibitor, purvalanol A, was used. After a 2 hour preincubation with 4 μM purvalanol A, PC3 cell migration was reduced by about 70% in a 4 hour migration assay. Please see attached figure 5 (note: not in the attached paper).

SOW Task 6. β3 antisense experiments (months 10-24):
1. Obtain pCEP-4 240 sense and 240 antisense plasmid contructs
2. Transfect PC-3 with plasmids
3. Select for hygromycin resistant clones
4. Pick and expand clones
5. Analyze clones by FACS analysis
6. Prepare extracts and analyze cdc2 levels by immunoblotting
7. Perform migration assays with selected clones
8. Test cells in SCID-human bone model

Not done.

SOW Task 7. Test cells infected with Adcdc2dn and AdFAKdn in SCID-human bone *in vivo* model (months 12-18):
1. Implant human bone fragments, wait 4 weeks
2. Inject transduced cells, wait 2 weeks
3. Inject adenoviral constructs into tumor site, wait 4 weeks

4. Harvest implants, measure growth of tumors, perform statistical analysis

Not done.

SOW Task 8. Identify the cyclin associated with the cyclin-cdc2dnHA complex in cdc2dnHA transfected β3-LNCaP cells (months 18-24):

1. Transfect β3-LNCaP cells with cdc2dnHA
2. Prepare extracts, immunoprecipitate with 12CA5
3. Run 12CA5 immunoprecipitates on gel, transfer, and immunoblot with cyclin antibodies and/or deplete 12CA5 immunoprecipitate with cyclin A and cyclin B antibodies, run on gel and stain with Coomassie or Silver, identify band, isolate, and microsequence

Different approaches were taken to identify the cyclin associated with "migratory" cdc2. First, migration assays of cells overexpressing cyclins A, B1, and B2 - the three cyclins known to associate with cdc2 – implicated cyclin B2. Second, cyclin B2 knockout cells migrated poorly in comparison to their wild-type counterparts, and this effect was independent of proliferation. Third, immunofluorescence co-localized cdc2 and cyclin B2 in membrane ruffles of migrating cells. Please see attached paper.

List of reportable outcomes:

Manes, T., Zheng., D.Q., Tognin, S., Woodard, A.S., Marchisio, P.C., Languino, L.R., $\alpha_v\beta_3$ integrin expression upregulates cdc2 which modulates cell migration. Submitted, Journal of Cell Biology.

Fornaro, M., Manes, T., Languino, L.R. Integrins and prostate cancer metastases. Cancer and Metastasis Reviews 20, 321-331, 2001.

Poster at The American Society for Cell Biology meeting, Washington, D.C., December 8-12, 2001.

$\alpha_V\beta_3$ Integrin Expression Upregulates cdc2 Which Modulates Cell Migration•

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Key words: cdc2, cell adhesion, cyclin B2, caldesmon, ?

Abbreviations: Ab, antibody; FN, fibronectin; VN, vitronectin; Ig, immunoglobulin

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Abstract

The $\alpha_v\beta_3$ integrin has been shown to promote cell migration through activation of intracellular signaling pathways. We describe here a novel pathway that modulates cell migration and that is activated by $\alpha_v\beta_3$ and, as downstream effector, by cdc2 (cyclin-dependent kinase 1, cdk1). We report that $\alpha_v\beta_3$ expression in LNCaP (β_3-LNCaP) prostate cancer cells causes increased cdc2 mRNA levels as evaluated by gene expression analysis, and increased cdc2 protein and kinase activity levels. We provide three lines of evidence that increased levels of cdc2 contribute to a motile phenotype on integrin ligands in different cell types. First, increased levels of cdc2 correlate with more motile phenotypes of several cancer cells. Second, ectopic expression of cdc2 increases LNCaP cell migration, whereas expression of dominant negative cdc2 inhibits migration. Third, cdc2 inhibitors reduce cell migration without affecting cell adhesion. We also show that cdc2 increases cell migration via specific association with cyclin B2 and we unravel a novel pathway of cell motility that involves, downstream of cdc2, caldesmon. Cdc2, cyclin B2 and caldesmon are shown here to localize in membrane ruffles in motile cells. These results show that cdc2 is a downstream effector of the $\alpha_v\beta_3$ integrin, and that it promotes cell migration.

Introduction

Cell-extracellular matrix (ECM) interactions are predominantly mediated by integrins [1]. The $\alpha_v\beta_3$ integrin is expressed *in vivo* predominantly, although not exclusively, in cancer cells and in neovessels (for review see [2, 3]). Expression of $\alpha_v\beta_3$ in tumor cells increases tumorigenicity as well as invasiveness [2].

Cell migration mediated by integrins, a crucial step in *in vivo* metastasis establishment and growth, has been shown *in vitro* to be supported by multiple downstream signaling pathways [4]. Although activation of these pathways is a prerequisite for cell migration, changes in gene expression are also likely to play a role in cell invasion. The increased invasive behavior of neoplastic cells that occurs in response to $\alpha_v\beta_3$ integrin expression can be explained on the basis of a unique $\alpha_v\beta_3$–activated cellular response that may positively regulate cell migration. We searched for downstream effectors of $\alpha_v\beta_3$ in prostate cancer cells where $\alpha_v\beta_3$ expression correlates with a neoplastic and migratory phenotype [5]. In this report, we show that $\alpha_v\beta_3$ integrin expression in LNCaP prostate cancer cells upregulates cdc2 mRNA and protein levels as well as cdc2 kinase activity. We demonstrate a new role for cdc2 in cell motility on integrin ligands and unravel a novel mechanism of cell motility mediated by cdc2, its cofactor cyclin B2 and, downstream of cdc2, caldesmon, a molecule known to be

associated with the cytoskeleton. Taken together, these data show that cdc2 is a downstream effector of $\alpha_v\beta_3$ and that it promotes cell migration.

Results

$\alpha_v\beta_3$ integrin upregulates cdc2 mRNA, protein and kinase levels

In an attempt to determine genes regulated by the $\alpha_v\beta_3$ integrin which contribute to this phenotype in cancer cells, a gene expression analysis was undertaken. As a model system, LNCaP prostate cancer cells were stably transfected with expression vector containing human β_3 integrin cDNA, or empty expression vector (mock), or expression vector containing human ICAM-1 cDNA as a transfection control for the effects of ectopically expressing a cell surface protein. Expression of $\alpha_v\beta_3$ integrin in three different cell populations (β_3-1, β_3-2, β_3-3), as well as ICAM expression in two different populations, was confirmed by FACS analysis (Fig. 1B and data not shown; see [5]).

First-strand cDNA of mRNA isolated from β_3-LNCaP, ICAM-LNCaP, and mock-LNCaP cells were used as probes on cDNA array filters containing 588 human genes known to be disregulated in cancer. Only those genes that displayed at least a three-fold difference in expression between β_3- and mock-LNCaP cells (Fig. 1A), or β_3- and ICAM-

LNCaP cells (data not shown) were considered as legitimate targets of $\alpha_v\beta_3$ integrin-mediated expression. Among others, cdc2 was specifically upregulated in β_3-LNCaP cells (Fig. 1A). Since cdc2 is a prognostic indicator of prostate tumor progression [6], further investigation of the expression of this gene was undertaken. Northern blot analysis was performed to verify the cDNA expression array results (data not shown).

Using extracts prepared from β_3-, ICAM- and mock-LNCaP cells we observed upregulation of cdc2 protein in β_3-LNCaP cells as compared to mock- and ICAM-LNCaP cells (Fig. 1C). Also shown in Fig. 1C are cdc2 levels in extracts from LNCaP cells stably transfected with an expression vector containing the human β_6 integrin subunit cDNA (β_6-LNCaP clones: β_6-1 and β_6-2 shown in Fig. 1E). The β_6 integrin subunit was chosen since, like β_3, it is not expressed in LNCaP cells (not shown) and it also heterodimerizes with α_v and shares several ligands with $\alpha_v\beta_3$ [7,8]. The results show that cdc2 levels in two clones expressing $\alpha_v\beta_6$ are significantly lower than in β_3-LNCaP cells. β_3-LNCaP cells display increased cdc2 kinase activity compared to mock- and ICAM-LNCaP cells (Fig. 1D) as well as β_6-LNCaP cells (not shown). cdc2 protein and kinase levels were also increased in three-dimensional (3D) Matrigel cultures (Figs. 1F and 1G, respectively) in β_3-LNCaP cells compared to mock- and ICAM-LNCaP cells. β_3-2 and mock-LNCaP 3D cell cultures appeared morphologically similar at 48 h, the time point selected for analysis (Fig. 1H).

Overall, the results show that the $\alpha_v\beta_3$ integrin expression in LNCaP cells specifically upregulates cdc2 mRNA, protein and kinase levels.

Correlation of cdc2 expression and cell migration

Since several molecules known to affect cell cycle progression or proliferation such as FAK and PI3K are positive modulators of cell migration [9-11], it was hypothesized that the increased cdc2 protein and kinase levels might play a role in LNCaP cell migration. A correlation between cdc2 levels and more migratory phenotypes on integrin ligands was observed. β_3-LNCaP cells migrated at a higher extent as compared to β_6-LNCaP cells on fibronectin (FN)-coated filters ($p= 2x10^{-5}$), although both cell types attached equally well to this substrate (Fig. 2A). This correlation was accentuated in another prostate cancer cell line (PC3) and its more metastatic variant (PC3MM2) [12], in which cdc2 levels were amplified to a greater extent, as was the migratory ability on FN ($p=1x10^{-9}$), under conditions in which these cells attached equally well to the substrate (Fig. 2B). Yet another correlation was seen between a human fibrosarcoma cell line (HT1080) and a genetically modified variant cell line (HT2-19) in which one cdc2 allele has been deleted, and the other placed under the control of an inducible promoter; removal of IPTG suppresses expression of the remaining allele [13]. Lower levels of cdc2 in HT2-19 cells correlated with a reduction in migration on FN ($p=2x10^{-8}$), under

conditions in which they attached equally well to FN (Fig. 2C). When HT2-19 cells were cultured in the absence of IPTG to suppress cdc2 expression, these cells' migration was further reduced (67% less than in the presence of IPTG, $p=2x10^{-5}$; Fig. 2C). The results show that increased levels of cdc2 correlate with a more migratory phenotype on integrin ligands.

cdc2 modulates LNCaP cell migration

Transient expression of cdc2 wild-type (cdc2wt) and a dominant negative variant of cdc2 (cdc2dn) was used to determine whether cdc2 had an effect on migration of LNCaP cells. Ectopic expression of either cdc2dn or cdc2wt affected migration of β_3-LNCaP cells on FN (Fig. 3A) and vitronectin and β_6-LNCaP cells on FN (not shown), **but had no effect on adhesion (not shown)**. Ectopic expression of dominant negative cdc2 (cdc2dn) and cdc2wild-type (cdc2wt) in LNCaP cells was efficiently achieved by lipofection and were indistinguishable (not shown). To establish whether these results were due to cdc2 effects on cell proliferation, β_3-LNCaP cells transfected with either cdc2wt or cdc2dn were tested in migration assays in the presence or absence of an inhibitor of cell proliferation, mitomycin c. As shown in Fig. 3A, mitomycin c inhibited cell proliferation, but had no effect on migration, in cells transfected with cdc2wt or cdc2dn. **Expression of cdc2dn**

also inhibited cell migration in conditions of low serum (Fig. 3B). These results show that cdc2 promotes cell migration on integrin ligands.

Two potent and specific inhibitors of cdc2 kinase, purvalanol A [14] and alsterpaullone [13],were tested to confirm a role for cdc2 in cell migration. After 2 h exposure to 0.2 or 1 μM purvalanol A, migration was reduced by more than 30 and 70%, respectively (Fig. 3C). Migration was reduced by about 50% in cells cultured in the presence of 1.32 μM alsterpaullone for 2 h (not shown). Neither adhesion nor cell morphology was significantly or noticeably affected by these concentrations of inhibitors, as determined by adhesion assays and microscopical analysis, respectively (Fig. 3C and not shown). In conclusion, inhibition of cdc2 kinase activity prevents cell migration without affecting cell adhesion.

Cdc2 modulates migration of HeLa cells

To investigate whether cdc2 regulates migration in cells other than LNCaP, cdc2dn and cdc2wt were ectopically expressed in HeLa cells and their effects on migration and cell cycle were determined over time. At 24 h, there is a modest reduction of HeLa cell migration on FN by cdc2dn and a two-fold increase in migration by cdc2wt (Fig. 4A). Since cdc2 activity is regulated by cyclin levels, we tested whether expression of cdc2 would be sufficient to increase kinase levels. Immunoprecipitation of cdc2 from cells

transfected with vector alone or cdc2wt determined that ectopic expression of cdc2wt increased cdc2 kinase activity (Fig. 4B). At 48 h, cdc2dn reduces HeLa cell migration more than 3-fold, and cdc2wt increases migration modestly (Fig. 4A); neither adhesion nor cell cycle profile was affected by either cdc2dn or by cdc2wt at these time points (not shown). HeLa cells cultured in the presence of micromolar concentrations of purv A for 2 h show a dose-dependent reduction of migration on FN with a negligible effect on adhesion at 8 μM (Fig. 4C). The results show that expression of cdc2wt and cdc2dn affects HeLa cell migration before a significant effect on cell cycle can be observed and that treatment with cdc2 inhibitors blocks HeLa cell migration.

Cdc2 is present in peripheral areas of the cell and in membrane ruffles

We investigated whether cdc2 is associated to the cytoskeleton of adhering cells and in cells with a motile phenotype. HeLa cells, plated on FN for 3 h, were incubated in the presence or absence of 100 nM PMA for 30 min to induce a motile phenotype (Fig. 4D; [19]). Staining of HeLa cells with cdc2 mAb and phalloidin shows that cdc2 is more concentrated in peripheral areas of the cell where bundles of actin microfilaments converge on adhesion areas (Fig. 4E). Upon PMA treatment, cdc2 tends to be even more concentrated at the periphery and often become very abundant in membrane ruffles, as determined by ezrin staining (Fig. 4E). Thus, cdc2 in resting and motile cells is

concentrated in peripheral areas where rapid actin reorganization occurs; in this cellular location, cdc2 may act on specific cytoskeleton proteins to modulate cell migration.

Cyclin B2 is the cyclin partner of cdc2 that modulates cell migration

Mammalian cdc2 is known to associate with cyclins A, B1, and B2 (reviewed in [20]). Ectopic expression of cyclin B2, but not cyclin A or cyclin B1, increased β_3-LNCaP and HeLa cell migration on FN (Fig. 5A), without affecting cell adhesion to this substrate (data not shown). The ectopically transfected cyclin B2 was capable of forming active kinase complex, as shown by immunoprecipitation kinase assays of non-transfected versus cyclin B2-transfected HeLa cells using a cyclin B2 antibody, as well as by immunoprecipitation kinase assay of cyclin B2 transfected HeLa cells using a c-myc antibody (Fig. 5B).

To confirm the role of cyclin B2 in cell migration, mouse embryonic fibroblasts (MEFs) derived from cyclin B2-null mice [21] were compared to their wt counterparts. Cyclin B2-null MEFs did not significantly migrate on FN (83% less than wt MEFs); adhesion to FN was similar for both cell types (Fig. 5C). These differences in migration were not due to an increased proliferation capacity of wt MEFs as compared to cyclin B2-null MEFs since cyclin B2-null MEFs proliferate more than wt MEFs (Fig. 5C). All

together, these data implicate cyclin B2 as the specific cyclin partner of cdc2 that modulates cell migration.

Caldesmon is a downstream effector of cdc2 in the cell motility pathway

Caldesmon, a previously identified mitotic substrate of cdc2 [24,25], appeared to be a reasonable candidate downstream of cdc2 in the cell motility pathway since it is found in membrane ruffles and its ability to bind actin is reduced upon phosphorylation by cdc2 [26]. We tested the migration of HeLa cells ectopically expressing cdc2wt in conjunction with either caldesmon wt or a variant form of caldesmon containing all seven of its cdc2 phosphorylation sites mutated ("7th mutant")[27]. Whereas caldesmon wt increased cdc2-mediated migration, the caldesmon "7th mutant" suppressed it in a dose-dependent manner (Fig. 6A) without affecting cell adhesion (data not shown). Expression of caldesmon wt and of the "7th mutant" was confirmed by IB analysis (data not shown). We next confirmed that caldesmon is a substrate of cdc2/cyclin B2 by immunoprecipitation kinase assay using caldesmon immunoprecipitate (cald IP) as a substrate for cyclin B2 immunocomplexes (B2 IP) (Fig. 6B). These results implicate cdc2 phosphorylation as an important mechanism by which caldesmon functions to modulate migration. This conclusion is also supported by the finding that cdc2 and caldesmon co-localize in all cells where membrane ruffles were observed; neither cdc2

nor caldesmon were found by themselves in membrane ruffles (Fig. 6C). In cells not treated with PMA, the two molecules did not co-localize (Fig. 6C); caldesmon was found in stress fibers, in agreement with previous studies [26, 27] (Fig. 6C). All together, these results show that caldesmon is a downstream effector of cdc2 in the cell motility pathway.

Discussion

In this study, we show that cdc2 is a downstream effector of the $\alpha_v\beta_3$ integrin, and that it controls cell migration. Evidence is provided that exogenous expression of $\alpha_v\beta_3$ integrin upregulates cdc2 mRNA and protein levels as well as cdc2 kinase activity and that cdc2 regulates cell migration on integrin ligands without affecting cell adhesion. It is also shown that cyclin B2 is the cdc2 cofactor that controls cell migration. Finally, it is shown that caldesmon, a cytoskeleton-associated molecule known to be phosphorylated by cdc2, is a substrate for cdc2/cyclin B2 in the migratory pathway, and co-localizes with cdc2 at the cell periphery and in membrane ruffles in motile cells.

This is the first report that describes changes in cdc2 levels in response to integrin expression. While regulation of cell cycle-related molecules in response to integrin engagement has been widely documented [28], regulation of the levels of a cdk and of its kinase activity upon integrin expression has never been reported. Specifically, alterations of cdc2 protein levels in response to either integrin expression or integrin engagement have never been studied. In one study, cdc2 mRNA levels were shown to remain unchanged in response to cell adhesion [29]. However, this analysis was performed in synchronized cells collected at times corresponding to transit through G1, whereas our study was performed in asynchronous cell populations. It should be noted that cdc2 protein levels do not vary significantly throughout the cell cycle [30-33] indicating that the

rise of cdc2 seen in asynchronous populations of β_3-LNCaP cells reflects a fundamental shift in cdc2 regulation by $\alpha_v\beta_3$.

To the best of our knowledge, this is the first report identifying cdc2 as a modulator of cell motility. Cdc2 is best characterized for its role in promoting cell cycle progression through the G2/M phase [34]. In our study, experimental conditions where either cdc2dn or cdc2 inhibitors affected cell migration but did not affect cell proliferation were established in all cell types; the long doubling time of LNCaP cells facilitated the initial discrimination between cell migration and cell proliferation.

We describe a novel motility mechanism that appears to be specifically activated by expression of $\alpha_v\beta_3$. This results in activation of a motility pathway that is not due to a specific integrin-ligand interaction. Our data show that expression of $\alpha_v\beta_3$ integrin, but not $\alpha_v\beta_6$ integrin, upregulates cdc2; however, in the cell types that were analyzed here, cdc2 affects migration on at least two different integrin substrates: VN (an $\alpha_v\beta_3$ ligand) and FN (an $\alpha_v\beta_3$, $\alpha_v\beta_6$ and $\alpha_5\beta_1$ ligand) [1]. However, cdc2 has an effect also in cells that do not express $\alpha_v\beta_3$, thus indicating that basal levels of cdc2 have the ability to promote cell migration on integrin ligands. In conclusion, upregulation of cdc2 levels by $\alpha_v\beta_3$ results in an increase of the cell's migratory potential that does not require a specific integrin-ligand interaction.

We demonstrate that the mechanism by which cdc2 regulates cell migration is via its specific association with cyclin B2. This claim is based on several experimental evidence: first, B2 and cdc2 co-localize in membrane ruffles of motile cells; this is a novel finding since previous reports had analyzed cdc2 distribution in non-motile cells and shown at interphase to be distributed both in the nucleus and the cytoplasm [35,36]. Second, ectopic expression of cyclins B1, B2 and A, all known to associate with cdc2 (reviewed in [37]) shows that only cyclin B2 has the ability to increase cell migration. Third, cyclin B2-null cells display significantly reduced cell migration, although their proliferation rates are not reduced. Finally, cyclin B2-associated kinase activity is detectable in G2-synchronized cells, which display a robust migration. Indeed, subcellular localization of mitotic cdc2 by cyclins B1 and B2 has been shown to confer substrate specificity [38]. Thus, it is conceivable that the cdc2-cyclin B2 complex will also provide specificity for the substrate(s) that modulates a non-mitotic event like cell migration. We reasoned that the cdc2-substrate that is likely to mediate cdc2's effect on cell motility would be co-localized with cdc2. Among the mitotic substrates for cdc2 that are known to be associated with the cytoskeleton: dynein, caldesmon, plectin, and zyxin [24,25,39-41], caldesmon was a strong candidate to mediate cdc2's effect on cell migration for two main reasons. First, caldesmon is associated with cytoskeletal structures such as stress fibers and membrane ruffles, and has been shown to interfere with focal contact

formation; second, its actin binding ability is reduced upon phosphorylation by cdc2 [26,27,42-44]. Our study shows that caldesmon, indeed, modulates cell motility downstream of cdc2 and that phosphorylation by cdc2 is a crucial step in this motility pathway. It also shows that caldesmon and cdc2 co-localize in membrane ruffles, sites of rapid reorganization of actin. In these sites, cdc2 phosphorylation of caldesmon may affect actin reorganization during cell migration by modulating caldesmon's actin-binding ability [24,42,43,45] and, potentially, focal contact formation [44].

Previous observations showed that $\alpha_v\beta_3$ expression is detected only in prostate cancer, but not in normal prostate epithelial cells [5]. Our data suggest that $\alpha_v\beta_3$ integrin and its downstream effector, cdc2, may be important mediators of prostate cancer progression towards an aggressive metastatic phenotype. This conclusion is supported by our data showing that more metastatic prostate cancer cell variants express higher levels of cdc2 and by data reported by Kallakury et al., indicating that cdc2 is expressed in a majority of prostatic adenocarcinomas and correlates with high Gleason's grade, advanced pathologic stage and metastatic adenocarcinomas [6]. Changes in gene expression during prostatic metastatic spread *in vivo* are likely to occur as recently shown for RhoC in melanoma cells [46]. In conclusion, the functional role of cdc2 in prostate cancer *in vivo* may be different than once thought; it may reflect the migratory, rather than the proliferative, ability of these cells.

Materials and Methods

Cells

LNCaP cell populations stably transfected with the pRc/CMV expression vector alone (mock) or containing human β_3 integrin cDNA (β_3-1) or ICAM-1 cDNA (ICAM) have been described [5]. In this study, two additional β3 populations (β_3-2 and β_3-3) and two β_6 LNCaP clones transfected with human β_6 integrin cDNA [47] were generated. Expression of $\alpha_v\beta_6$ integrin was confirmed by FACS analysis using the 10D5 mAb; mouse IgG was used as negative control. HeLa, HT1080 and PC3 cells were obtained from ATCC. PC3MM2 cells [12] were donated by Isaiah J. Fidler (The University of Texas MD Anderson Cancer Center, Houston, TX). HT2-19 cells [13] were a gift of Dr. Andrew C.G. Porter (MRC, London, UK). Cyclin B2-null and wt MEFs [21] were provided by Dr. Tim Hunt (ICRF, South Mimms, Herts, UK). For 3D cultures, cells were resuspended in Matrigel at a cell density of 10^6 cells/ml. For biochemical analysis, 3D cultures were detached from Matrigel by incubating with Matrisperse. For biochemical analysis or gene expression profiles, 2D cultures were detached by trypsinization or, in parallel with

the 3D cultures, by using Matrisperse (1 h on ice). Alternatively, 2D cultures were lysed directly on the plate.

Cell adhesion assays

Adhesion assays were performed as previously described [50].

RNA isolation and analysis

Gene expression profiles of β_3-1, ICAM and mock LNCaP cells were generated using Atlas Human Cancer cDNA Expression arrays (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Northern blot analysis was performed using total RNA from 2D and 3D cell cultures isolated using TRIzol Reagent (Gibco BRL). The 231 bp cdc2 cDNA fragment corresponding to the fragment on the cDNA array was generated by PCR. The cDNA fragment was amplified using the primers 5'-GGGTCAGCTCGTTACTCAACTCCAG-3' and 5'-GACATGGGATGCTAGGCTTCCTGGT-3' and human cdc2 cDNA as template. A 780 bp human GAPDH cDNA was excised from pGEM-3zf(+) with BamHI and PstI.

Immunoblotting and *in vitro* kinase assays

Cells were lysed using RIPA buffer (with 50 mM sodium fluoride for lysates to be used in *in vitro* kinase assays), either directly on the plate after washing with PBS, or after detaching with Matrisperse, as described for the 3D cultures above. For HT1080 and HT2-19 cells, cells were lysed as described [13].

Primary antibodies (Abs) to cdc2 (mAb, sc-54), to c-myc (mAb 9E10 and rabbit polyclonal agarose conjugate), to cyclin B2 (goat polyclonal sc-5235) and to ERK-1 (rabbit polyclonal that cross-reacts with ERK-2, sc-94) were from Santa Cruz Biotechnology. Rabbit antiserum to cyclin B2 was a kind gift from Jonathon Pines. Caldesmon mAb SM12 was a kind gift from Fumio Matsumura. Immunoprecipitation of cdc2 and *in vitro* kinase assays were performed essentially as described [30,51-53]. 60 or 75 µg pre-cleared cell lysate was used for immunoprecipitation using 2 µg mAb sc-54 or mouse IgG for 1 h, followed by protein A-sepharose (Sigma Fast Flow) for 1 h. After two washes with lysis buffer and one wash with kinase buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM sodium orthovanadate, 0.1 mM sodium fluoride), immunoprecipitates were incubated in 20 µl kinase buffer with 250 µg/ml histone H1, 25 µM ATP, 62.5 µCi/ml [γ-^{32}P]-ATP for 30 min at 30°C, stopped by the addition of loading buffer, heated at 98°C for 5 min, separated on 12% SDS-PAGE, and visualized by autoradiography.

For cyclin B2 immunoprecipitation-kinase assays, cyclin B2 was immunoprecipitated from HeLa RIPA extracts with a cyclin B2 rabbit polyclonal antibody as described [23] and incubated with histone H1 as described above, or with caldesmon immunoprecipitated from HeLa cells. Caldesmon was immunoprecipitated as described [54], except that, since caldesmon is heat stable, the lysate was boiled and clarified by centrifugation before immunoprecipitation by mAb SM12. Specifically, 5 x 10^6 HeLa cells were lysed with 50 mM Hepes, pH 7.5, 1% Triton X-100, 1% NP-40, 0.5% deoxycholate, 50 mM NaCl, 5 mM EDTA, 0.1 mM sodium vanadate, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, centrifuged 14,000 g for 10 min. The supernatant was then boiled for 5 min, cooled on ice 30 min, and centrifuged 14,000 g 10 min. An equal volume of immunoprecipitation buffer A (2.5% Triton X-100, 50 mM Tris-HCl pH 7.4, 6 mM EDTA, 190 mM NaCl) was then added to the supernatant which was then pre-cleared with protein A-Sepharose. Caldesmon mAb SM12 was then added to the pre-cleared lysate. After 1 h on ice, protein A-Sepharose was added and samples were rocked at 4°C for 1 h. Immunoprecipitates were washed 3 times with buffer B (150 mM NaCl, 10 mM Tris-HCl pH 9, 5 mM EDTA, 0.1% Triton X-100) and once with kinase buffer (described above). Caldesmon immunoprecipitates were then used as substrate for either cyclin B2 immunocomplexes or recombinant cdc2/cyclin B1 (New England Biolabs).

Migration Assays

β_3-LNCaP, β_6-LNCaP, HeLa, HUVECs and PC3 cells were transiently co-transfected with a 1:7 ratio of pCMV- βgal and pcDNA-3 (empty vector), pCMVcdc2dn-HA, or pCMVcdc2wt-HA [14]. β_3-LNCaP and HeLa cells were also transfected with pCMV-βgal and pCMX cyclin A, pCMV cyclin B1, or pCMV cyclin B2. HeLa cells were also transfected with pCMV-βgal and pCMVcdc2wt-HA and pCMV rat non-muscle caldesmon wt or pCMV rat non-muscle caldesmon "7th mutant" [27]. For the titration of caldesmon "7th mutant", the following amounts of empty vector and pCMV caldesmon "7th mutant" were used (0 μg vector plus 3μg pCMV caldesmon "7th mutant"; 2.67μg vector plus 0.33 μg pCMV caldesmon "7th mutant"; 2.97 μg vector plus 0.03μg pCMV caldesmon "7th mutant"). Lipofectamine 2000 (Gibco/BRL) was used as the transfection reagent. One to three days after transfection, the cells were seeded on 8 μm pore-sized transwell filter inserts (Costar) coated with 5 or 10 μg/ml FN or 3 μg/ml VN. In parallel, transiently transfected cells were also seeded on FN, VN, and poly-L-lysine-coated plates to measure their ability to adhere to these substrates. After 6 h (LNCaP) or 16 h (HeLa), cells were fixed with 0.2% glutaraldehyde, washed with TTBS, and stained for βgal using x-gal as substrate (400 μg/ml x-gal, 0.5mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$ in PBS), at 37°C for 2 h. The number of transfected cells in 10 random fields on

the top and the bottom were counted for each filter. The percentage (average and s.e.m.) of the attached transfected cells (βgal positive cells on the top and bottom of the filter) that migrated (βgal positive cells on the bottom of the filter) was calculated.

β_3-LNCaP, β_6-LNCaP, PC3, PC3MM2, HT1080, HT2-19 cells, cyclin B2-null and wt MEFs were seeded on 5 μm (HT1080, HT2-19), 8 μm (β_3-LNCaP, β_6-LNCaP), or 12 μm (PC3, PC3MM2, cyclin B2-null MEFs, wt MEFs) pore-sized transwell filter inserts coated with 5 or 10 μg/ml FN or 3 μg/ml VN. After 4 h (PC3, PC3MM2, HT1080, HT2-19, cyclin B2-null MEFs, wt MEFs) or 6 h (β_3-LNCaP, β_6-LNCaP), cells were fixed with 3% paraformaldehyde/PBS, stained with crystal violet, and the number of cells per square millimeter on the bottom were counted (average and s.e.m of 10 random fields).

For cells cultured in the presence or absence of alster and purv A (Calbiochem) for 2 h, cells were seeded on filters as above in the absence or presence of alster or purv A, for 6 h (β_3-LNCaP) or 16 h (HeLa), and counted as described above. In parallel, cell adhesion assays in the presence of alster and purv A were performed; cells were seeded in 96-well plates coated with 1-10 μg/ml FN or 3μg/ml VN for 2 h, fixed with 3% paraformaldehyde/PBS, stained with crystal violet, and the absorbance at 630 nm measured.

For cells cultured in the presence of mitomycin C (Sigma)(16 h incubation), cells were trypsinized and seeded on filters as above in the absence or presence of mitomycin C. After 6 h, cells were stained for βgal and the number of cells per square millimeter on the top and bottom were counted (average and s.e.m of 10 random fields).

Proliferation assays

For cells cultured in the presence of mitomycin C (Sigma)(16 h incubation), cells were trypsinized and seeded on FN-coated 96-well plates, in triplicate, in the presence of 1 μCi [^{3}H]-Thymidine in 100 μl medium per well (in the presence or absence of mitomycin C) and incubated for 6 h. Medium was then removed, and cells were washed three times with PBS, solubilized with 10% SDS, and the amount of [^{3}H]-Thymidine incorporated by the cells was quantitated by scintillation counting.

Immunofluorescence microscopy

HeLa cells were seeded on 10 μg/ml FN-coated coverslips, incubated in the presence or absence of 100 nM PMA, fixed with 3% paraformaldehyde, 2% sucrose, pH 7.6 for 10 min at room temperature. Coverslips were stained with fluorescein-tagged phalloidin and mAb to cdc2 (A17, IgG2kappa, Zymed), mAb to L-caldesmon (Clone 8, IgG1, BD Transduction Laboratories) and mAb to ezrin (Clone 18, IgG1, BD Transduction

Laboratories) in association with mAb to cdc2. Secondary Abs were class-specific goat anti-mouse IgGs coupled to either FITC or Texas Red (Southern Biotechnologies). In some single staining experiments, we also used a different mAb to caldesmon (SM12) and obtained essentially identical results. Non-binding mouse IgGs were used as a control. Nuclear staining was performed with Hoechst 33342. For cyclin B2, goat polyclonal Ab to cyclin B2 (Santa Cruz Biotechnology) and, as secondary Ab, a FITC-rabbit anti-goat (DAKO) were used. Two hundred cells were systematically analyzed and approximately 30% cells showed changes in morphology upon PMA treatment.

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Figure Legends

Figure 1 **Cdc2 mRNA, protein, and kinase levels are increased in β_3-LNCaP cells.** (A) cDNA expression array analysis. First strand cDNA probes prepared with either β_3-1 or mock-LNCaP mRNA were hybridized to Atlas Human Cancer cDNA Expression arrays. Shown are areas of panel A of the cDNA array membrane. The arrow indicates the spots corresponding to cdc2 on the array. The brackets indicate other genes on the array that are not upregulated in β_3-1. (B) Surface expression of ectopic $\alpha_v\beta_3$ integrin in LNCaP cell stable transfectants. Mock-, β_3-1, β_3-2 and β_3-3 LNCaP cells were incubated with mAb to $\alpha_v\beta_3$ integrin (AP-3, thick line) or, as negative control, to the HA epitope (12CA5, thin line) followed by staining with FITC-conjugated goat anti-mouse Ig Ab and analysis using a FACScan^{TM} flow cytometer. (C-H) Cdc2 protein and kinase levels are increased in β_3-LNCaP cells grown in 2D (C-E) and 3D (F-H) cultures. (C) cdc2 protein levels in extracts from LNCaP cell transfectants grown in 2D culture were analyzed by IB with an Ab to cdc2. In C and F, Ab to ERK-1 was used for protein loading control. (D) Immunocomplexes precipitated from 75 μg of β_3-1, β_3-3, β_3-2, ICAM- or mock-LNCaP cell extracts using a mAb to cdc2 were used in a kinase reaction using histone H1 as a substrate. Consistent results were obtained in two additional experiments. (E) Surface expression of ectopic $\alpha_v\beta_6$ integrin in LNCaP cell stable transfectants. β_6-1 and β_6-2

LNCaP cells were incubated with mAb to $\alpha_v\beta_6$ integrin (10D5, empty peak) or mouse IgG as negative control (filled peak), followed by staining with FITC-conjugated goat anti-mouse Ig Ab and analysis using a FACScan™ flow cytometer. (F) cdc2 protein levels in extracts from LNCaP cell transfectants grown in 3D Matrigel culture for 48 h were analyzed by IB with an Ab to cdc2. (G) Immunocomplexes precipitated from 75 μg of β_3-1, β_3-2, ICAM- or mock-LNCaP cell extracts using a mAb to cdc2 were used in a kinase reaction using histone H1 as a substrate. Consistent results were obtained in two additional experiments. (H) Phase contrast photomicrographs of β_3-2 LNCaP and mock-LNCaP cells grown in 3D cultures for 48 h. Magnification X 200.

Figure 2 **cdc2 levels correlate with cell migration.** (A) β_3- and β_6-LNCaP cells. β_3-LNCaP (β_3-2) or β_6-LNCaP (β_6-2) cells were trypsinized and washed. For migration assays (left panel), 200,000 cells were seeded in medium containing 10% FBS on 6 μg/ml FN-coated transwell insert filters. After 6 h, cells were fixed and stained with crystal violet and the cells on the bottom of the filter were counted. The mean and s.e.m. of 10 random fields is shown (*$p=2x10^{-5}$). For adhesion assays (right panel), 100,000 cells were seeded in medium containing 10% FBS in a 96-well plate coated with 6 μg/ml FN. After 2 h, plates were washed two times with PBS, fixed, and stained with crystal violet. (B) PC3 and PC3MM2 cells. cdc2 protein levels in extracts from PC3 and

PC3MM2 cells were analyzed by IB with an Ab to cdc2 (top left panel). In (B) and (C), Ab to ERK-1 was used for protein loading control. For migration assays (top right panel), 300,000 cells were seeded in serum-free medium on 5 μg/ml FN-coated transwell insert filters. After 3.5 h, cells were fixed and stained with crystal violet and the cells on the bottom of the filter were counted. The mean and s.e.m. of 10 random fields is shown (**$p=1x10^{-9}$). For adhesion assays (bottom panels), 100,000 cells were seeded in serum-free medium in a 96-well plate coated with increasing concentrations of FN. After 2 h, plates were washed two times with PBS, fixed, and stained with crystal violet. (C) HT1080 cells, HT2-19 cells +IPTG, and HT2-19 cells -IPTG. cdc2 protein levels in extracts from HT1080 and HT2-19 cells were analyzed by IB with an Ab to cdc2 (top left panel: HT1080 and HT2-19 +IPTG; middle left panel: HT2-19 +/-IPTG). For migration assays (top and middle right panels), 50,000 cells were seeded for 4.5 h, as described in (B). The mean and s.e.m. of 10 random fields is shown (**$p=2x10^{-8}$ for HT1080 compared to HT2-19 cells +IPTG; *$p=2x10^{-5}$ for HT2-19 +IPTG compared to HT2-19 cells -IPTG). Adhesion assays (bottom panels), were performed as described in (B) using 50,000 cells.

Figure 3 **cdc2 affects migration of β_3-LNCaP cells.** (A) β_3-LNCaP (β_3-2) cells cotransfected with pCMVβgal and pcDNA-3 or pCMVcdc2wt for 8 h (top two panels) or

cotransfected with pCMVβgal and pcDNA-3 or pCMVcdc2dn for 60 h (bottom two panels) were incubated in the presence or absence of 1 μg/ml mitomycin C for 16 h, then harvested and seeded in medium containing 10% FBS with or without 1 μg/ml mitomycin C on 10 μg/ml FN-coated transwell insert filters or on 10 μg/ml FN-coated 96-well plates with 1 μCi [^{3}H]-Thymidine per well. After 6 h, cells were fixed and stained for βgal activity. The number of transfected cells that migrated and cells that proliferated was calculated as described in Methods. (B) β_3-LNCaP (β_3-2) cells cotransfected with pCMVβgal and pcDNA-3 or pCMVcdc2dn for 60 h were harvested and seeded in medium containing 0.5% FBS on 10 μg/ml FN-coated transwell insert filters. After 6 h, cells were fixed and stained for βgal activity and counted as in A. (C) Top panel: Purv A prevents β_3-LNCaP cell migration. β_3-LNCaP cells were incubated for 2 h at 37°C in the presence or absence of purv A and 200,000 cells were seeded in medium containing purv A at the indicated concentrations on 10 μg/ml FN-coated transwell insert filters. After 6 h, cells were fixed and stained with crystal violet and the cells on the bottom of the filter were counted. The mean and s.e.m. per square millimeter is shown. Bottom panel: Purv A does not affect β_3-LNCaP cell adhesion to FN. β_3-LNCaP cells incubated at 37°C in the presence or absence of purv A for 2 h were trypsinized, washed, and 50,000 cells were seeded in medium containing purv A at the

indicated concentrations in a 96-well plate coated with FN. After 2 h, plates were washed two times with PBS, fixed, and stained with crystal violet.

Figure 4 **Cdc2 regulates migration of HeLa cells.** (A) Expression of cdc2dn or cdc2wt affects migration of HeLa cells. HeLa cells cotransfected with pCMVβgal and pcDNA-3, pCMVcdc2dn, or pCMVcdc2wt were trypsinized at two different time points (24 h, 48h) after transfection and cells were seeded in medium containing 10% FBS on 10 μg/ml FN-coated (HeLa). After 16 h, cells were fixed and stained for βgal activity. (B) Expression of cdc2 wt increases cdc2 kinase activity. Immunocomplexes precipitated from 60 μg cell extracts of HeLa cells transfected with pcDNA-3 or cdc2 wt using a mAb to cdc2 were used in a kinase reaction using histone H1 as a substrate. (C) Purv A inhibits HeLa cell migration without affecting cell adhesion. Migration assays (upper panels): HeLa cells were incubated for 2 h at 37°C in the presence or absence of purv A and 90,000 HeLa cells were seeded in medium containing purv A at the indicated concentrations on FN-coated transwell insert filters. After 16 h, cells were fixed and stained with crystal violet and the cells on the bottom of the filter were counted. The mean and s.e.m. of 10 random fields is shown. Adhesion assays (lower panels): 100,000 HeLa cells grown in the presence or absence of purv A for 2 h were seeded in medium containing purv A at the

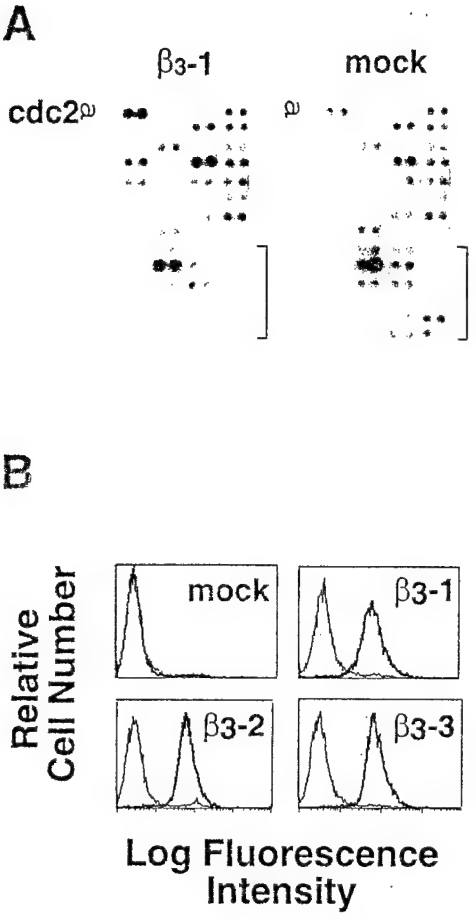
indicated concentrations in a 96-well plate coated with FN (HeLa). After 2 h, plates were washed two times with PBS, fixed, and stained with crystal violet. The results show the mean and standard deviation of duplicate observations. (D) PMA increases HeLa cell migration. HeLa cells were allowed to attach for 1 h to filters coated with 10 μg/ml FN and then were incubated in the presence or absence of 100 nM PMA for 3 h and processed as described above. (E) Cdc2 is enriched in peripheral areas in motile cells. HeLa cells plated on 10 μg/ml FN-coated coverslips and incubated in the presence of 100 nM PMA were stained with ezrin mAb (green) and cdc2 mAb (red). Right panels: image-merging analysis.

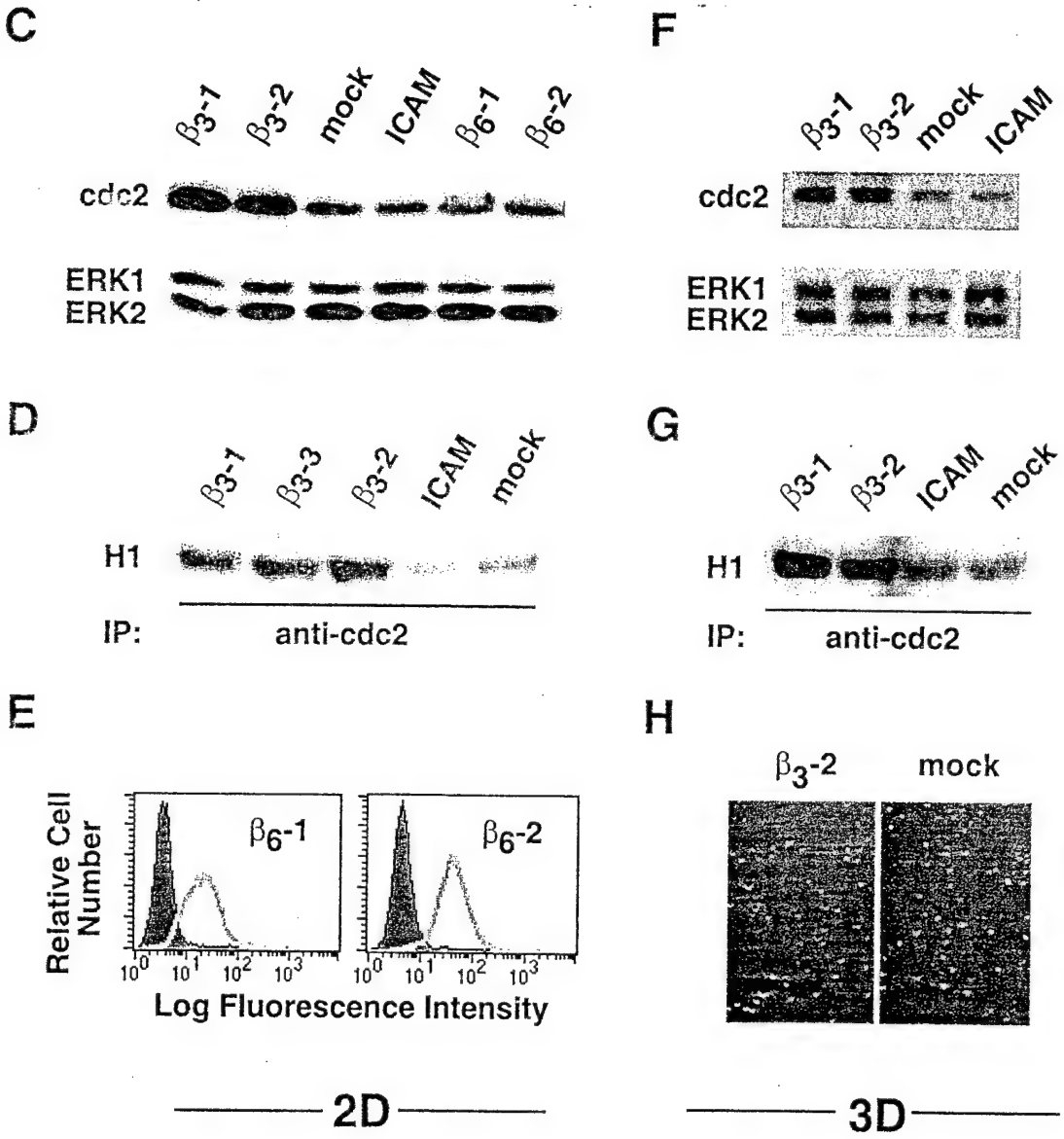
Figure 5 **Expression of cyclin B2 increases cell migration.** (A) β_3-LNCaP (β_3-2) and HeLa cells cotransfected with pCMVβgal and pcDNA-3 (-), pCMXcyclin A (A), pCMVcyclin B1 (B1), or pCMVcyclin B2 (B2) were processed 24 h after transfection, as described in Figures 3 and 4 (B) Ectopically expressed cyclin B2 forms active kinase complex. Immunocomplexes precipitated from HeLa and cyclin B2-transfected HeLa RIPA extracts using non-immune rabbit serum (n.i.), or rabbit polyclonal Ab to cyclin B2 (B2) or a c-myc agarose-conjugated rabbit polyclonal Ab (c-myc) were used in kinase assays using histone H1 as a substrate. (C) Cyclin B2-null cells migrate poorly on FN. For migration assays (top left panel), 15,000, 30,000 or 60,000 cyclin B2-null (B2 -/-)

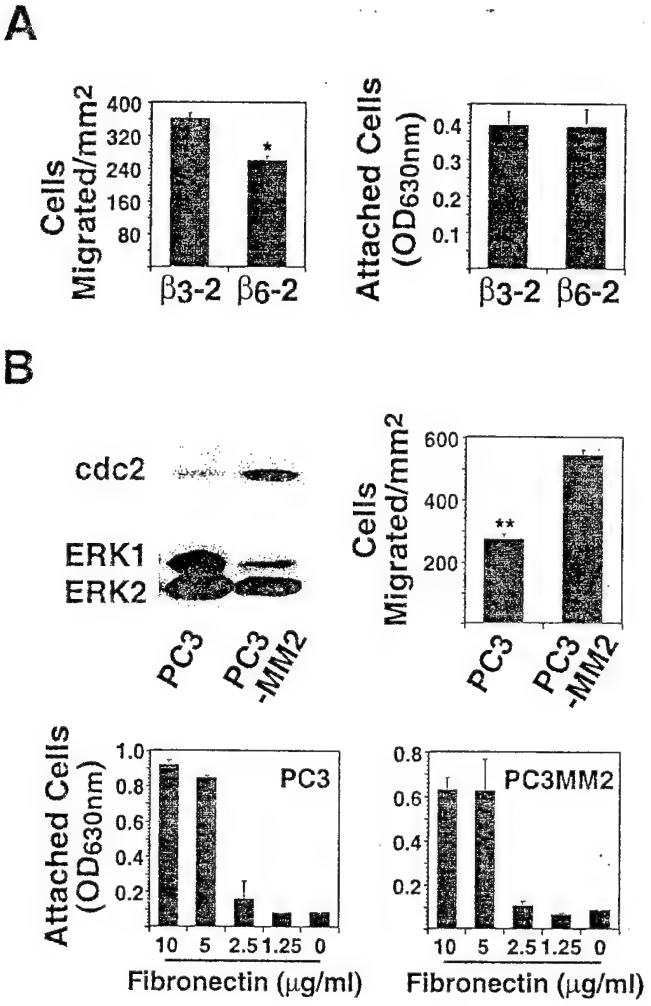
and wt (B2 +/+) MEFs were seeded in serum-free medium on 5 μg/ml FN-coated transwell insert filters. After 4 h, cells were fixed and stained with crystal violet and the cells on the top and bottom of the filter were counted. The mean and s.e.m. of 10 random fields is shown. For proliferation assays (top right panel), 10,000 cells were seeded in serum-free medium on 5 μg/ml FN-coated 96-well plates in the presence of 1 μCi [^{3}H]-Thymidine per well. After 4 h, cells were processed to determine [^{3}H]-Thymidine incorporation, as described in Methods. For adhesion assays (bottom panels), 50,000 cells were seeded for 2 h in serum-free medium in a 96-well plate coated with increasing concentrations of FN.

Figure 6 **Caldesmon is a downstream effector of cdc2 in the cell motility pathway.** (A) Co-expression of cdc2 and caldesmon wt increases, whereas caldesmon "7th mutant" decreases migration of HeLa cells. HeLa cells co-transfected with pCMVβgal and pCMVcdc2wt and empty vector or pCMV myc-tagged caldesmon or three different concentrations (see Methods) of pCMV myc-tagged caldesmon with seven mutated cdc2 phosphorylation sites ("7th mutant") were processed as in Fig. 5A 24 h after transfection. (B) Caldesmon is a substrate of cdc2/cyclin B2. (left panel) Caldesmon immunoprecipitated (cald IP) from HeLa cells or H1 was used as substrate of either

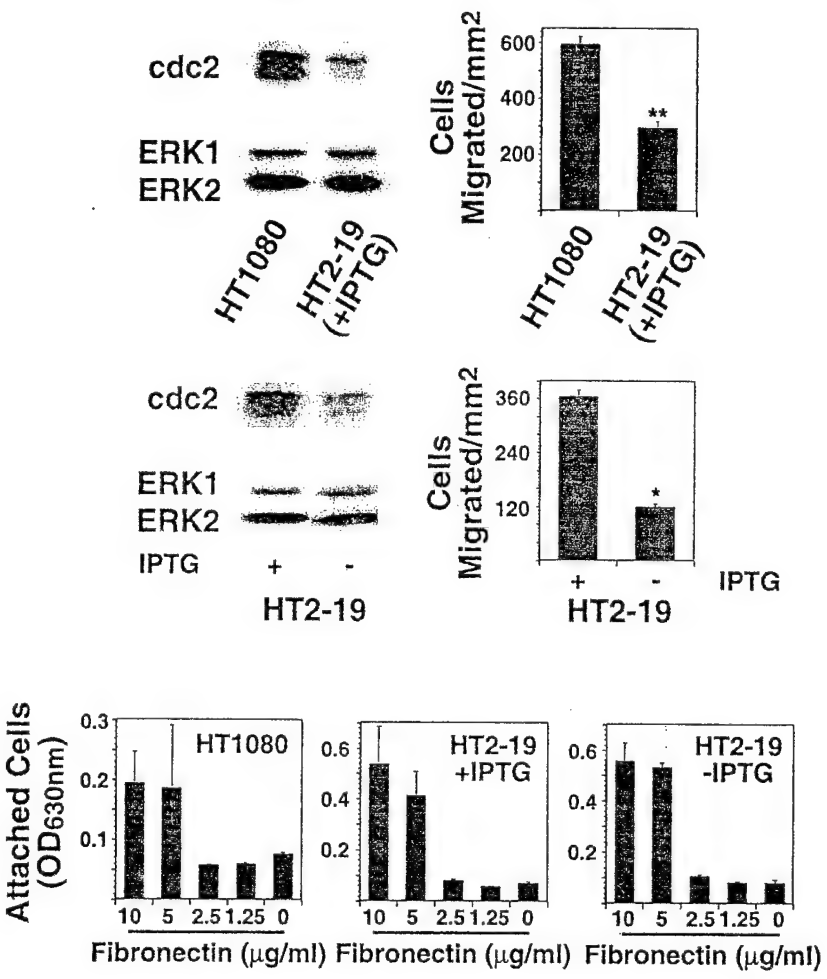
recombinant cdc2/cyclin B1 or cyclin B2 immunocomplexes in kinase assays. (middle panel) An equivalent amount of caldesmon immunoprecipitate used in the kinase assay, and 3 μg histone H1, were separated using SDS-PAGE and stained with Coomassie. (right panel) Immunoblot of caldesmon immunoprecipitate using SM12 mAb to caldesmon. (C) HeLa cells plated on 10 μg/ml FN-coated coverslips and incubated in the presence or absence of 100 nM PMA were stained with caldesmon mAb and cdc2 mAb. Image-merging analysis of caldesmon (green) and cdc2 (red) indicates yellow patches where superposition of the two molecules is particularly noted (see also arrows).

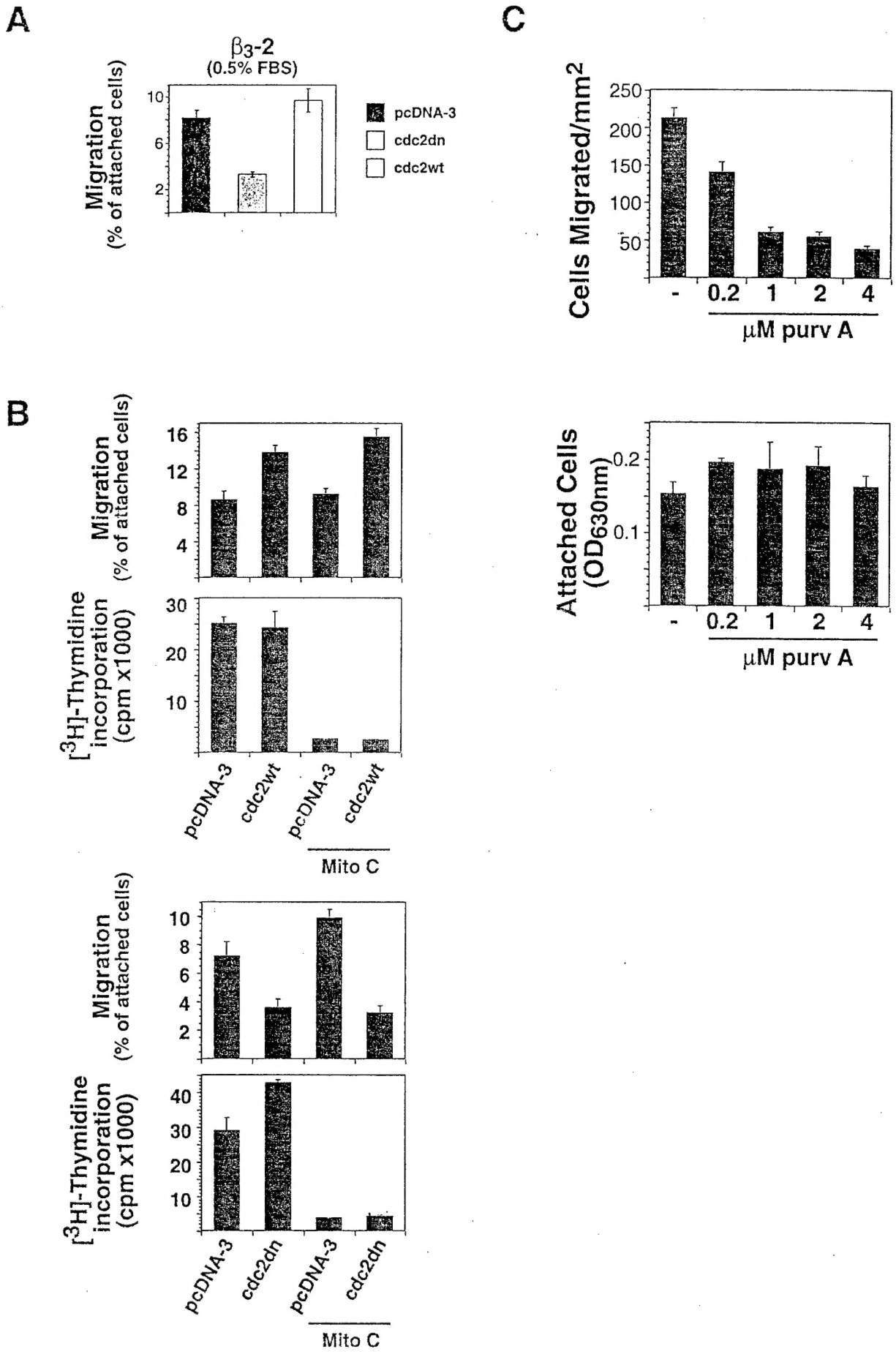

A
β3-1
mock
cdc2℗
℗
B
Relative Cell Number
mock
β3-1
β3-2
β3-3
Log Fluorescence Intensity

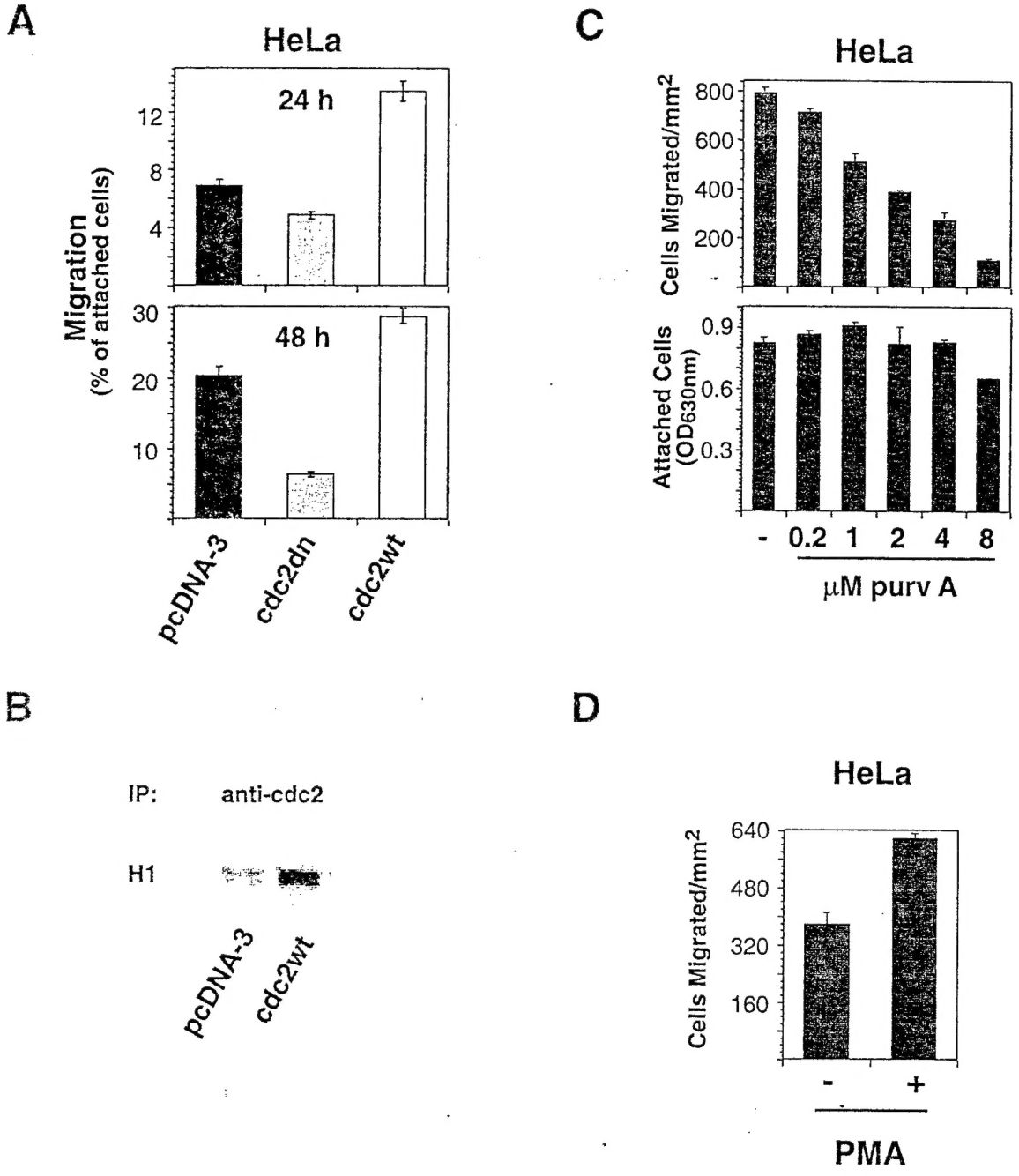

C
β3-1
β3-2
mock
ICAM
β6-1
β6-2
cdc2
ERK1
ERK2
F
β3-1
β3-2
mock
ICAM
cdc2
ERK1
ERK2
D
β3-1
β3-3
β3-2
ICAM
mock
H1
IP:
anti-cdc2
G
β3-1
β3-2
ICAM
mock
H1
IP:
anti-cdc2
E
Relative Cell Number
β6-1
β6-2
Log Fluorescence Intensity
H
β3-2
mock
2D
3D

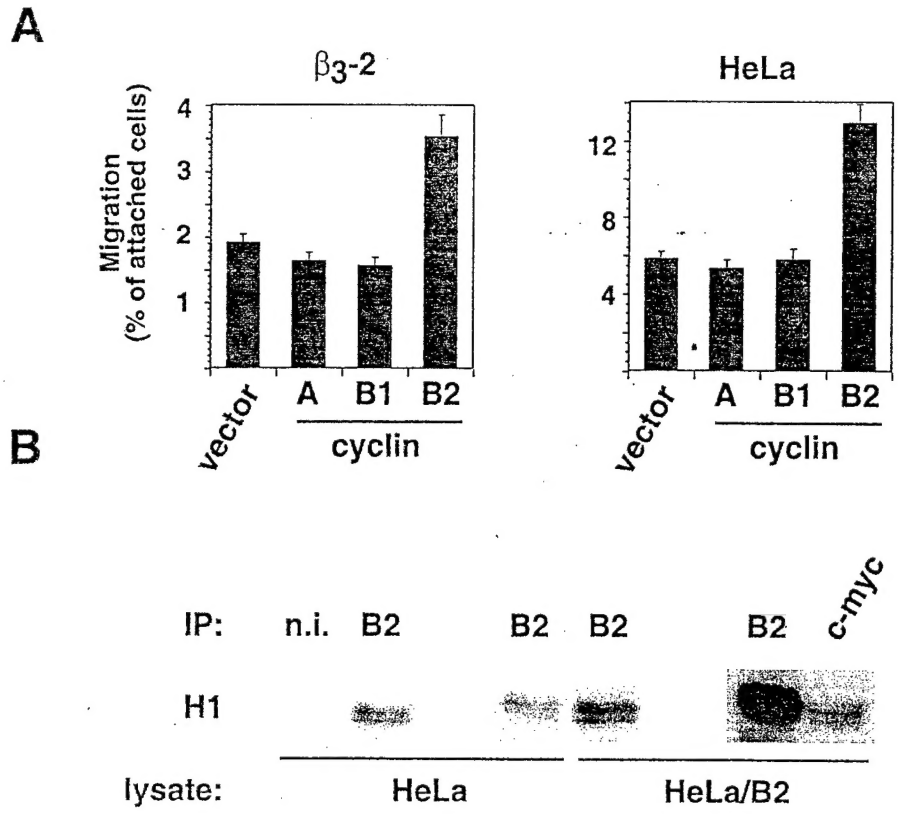

A
Cells Migrated/mm2
400
320
240
160
80
*
β3-2 β6-2
Attached Cells (OD630nm)
0.4
0.3
0.2
0.1
β3-2 β6-2
B
cdc2
ERK1
ERK2
PC3
PC3 -MM2
Cells Migrated/mm2
600
400
200
**
PC3
PC3 -MM2
Attached Cells (OD630nm)
PC3
1.0
0.8
0.6
0.4
0.2
10 5 2.5 1.25 0
Fibronectin (μg/ml)
PC3MM2
0.8
0.6
0.4
0.2
10 5 2.5 1.25 0
Fibronectin (μg/ml)

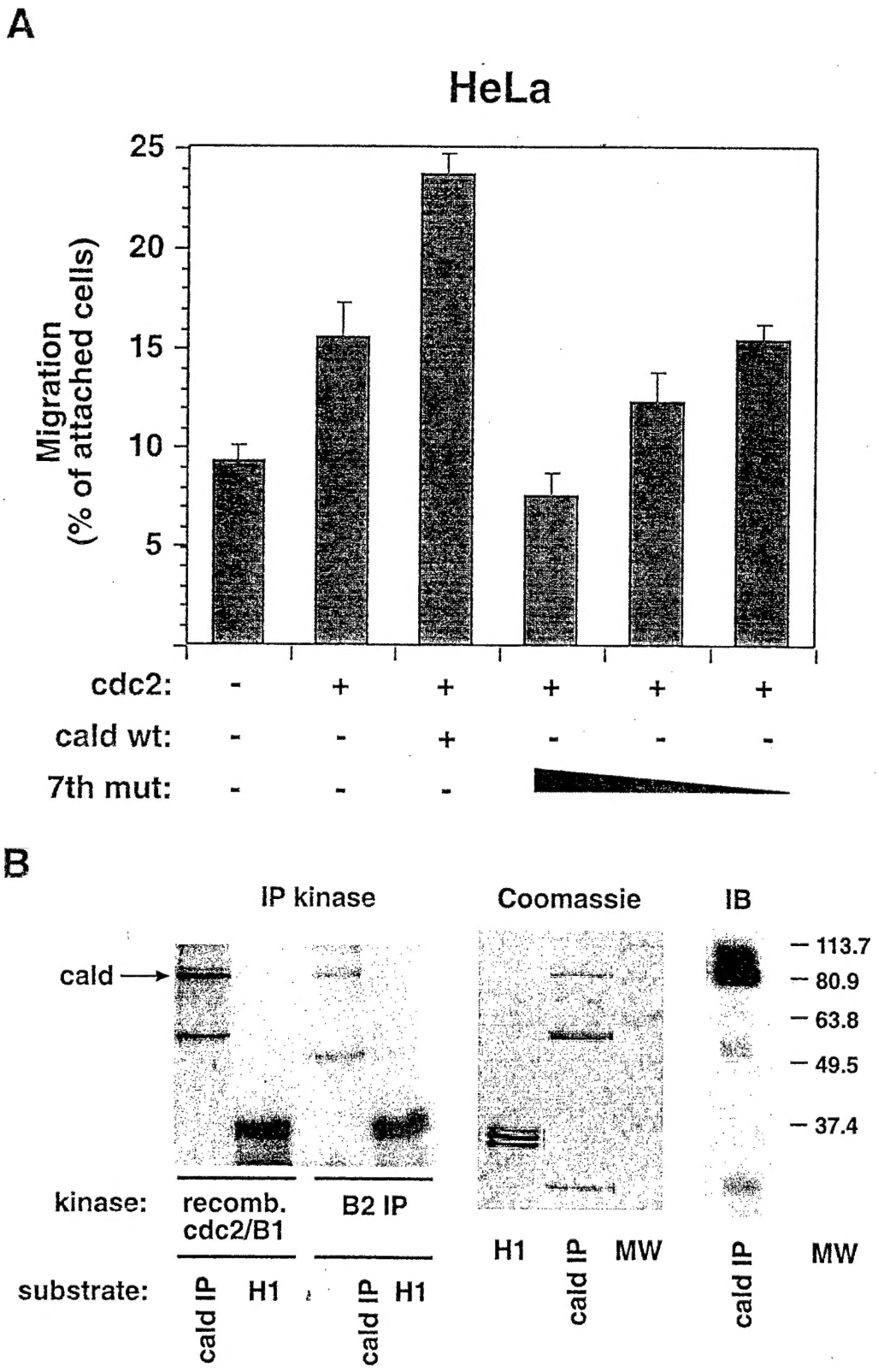
C


cdc2
ERK1
ERK2
HT1080
HT2-19 (+IPTG)
Cells Migrated/mm2
600
400
200
**
HT1080
HT2-19 (+IPTG)
cdc2
ERK1
ERK2
IPTG + -
HT2-19
Cells Migrated/mm2
360
240
120
*
+ - IPTG
HT2-19
Attached Cells (OD630nm)
HT1080
0.3
0.2
0.1
10 5 2.5 1.25 0
Fibronectin (μg/ml)
HT2-19 +IPTG
0.6
0.4
0.2
10 5 2.5 1.25 0
Fibronectin (μg/ml)
HT2-19 -IPTG
0.6
0.4
0.2
10 5 2.5 1.25 0
Fibronectin (μg/ml)


A
β3-2
(0.5% FBS)
Migration
(% of attached cells)
10
6
2
pcDNA-3
cdc2dn
cdc2wt
B
Migration
(% of attached cells)
16
12
8
4
[3H]-Thymidine
incorporation
(cpm x1000)
30
20
10
pcDNA-3
cdc2wt
pcDNA-3
cdc2wt
Mito C
Migration
(% of attached cells)
10
8
6
4
2
[3H]-Thymidine
incorporation
(cpm x1000)
40
30
20
10
pcDNA-3
cdc2dn
pcDNA-3
cdc2dn
Mito C
C
Cells Migrated/mm2
250
200
150
100
50
-
0.2
1
2
4
μM purv A
Attached Cells
(OD630nm)
0.2
0.1
-
0.2
1
2
4
μM purv A


A
HeLa
24 h
48 h
Migration
(% of attached cells)
12
8
4
30
20
10
pcDNA-3
cdc2dn
cdc2wt
C
HeLa
Cells Migrated/mm2
800
600
400
200
Attached Cells
(OD630nm)
0.9
0.6
0.3
- 0.2 1 2 4 8
μM purv A
B
IP: anti-cdc2
H1
pcDNA-3
cdc2wt
D
HeLa
Cells Migrated/mm2
640
480
320
160
- +
PMA


A
β3-2
HeLa
Migration (% of attached cells)
vector
A
B1
B2
cyclin
B
IP:
n.i.
B2
B2
B2
B2
c-myc
H1
lysate:
HeLa
HeLa/B2
C
Migration (% of attached cells)
[3H]-Thymidine incorporation (cpm x1000)
B2 +/+
B2 -/-
Attached Cells (OD630nm)
B2 +/+
B2 -/-
20 10 5 2.5 1.2 0
Fibronectin (μg/ml)


A
HeLa
Migration
(% of attached cells)
25
20
15
10
5
cdc2: - + + + + +
cald wt: - - + - - -
7th mut: - - -
B
IP kinase
Coomassie
IB
cald
113.7
80.9
63.8
49.5
37.4
kinase: recomb. cdc2/B1
B2 IP
substrate: cald IP
H1
cald IP
H1
H1
cald IP
MW
cald IP
MW